48, 87-59-2; 49, 6579-44-8; 50, 20205-43-0; 51, 117571-22-9; 52, 129833-49-4; 53, 117571-18-3; 54, 117571-19-4; 55, 117571-20-7; 2-(bromomethyl)-4-methyl-1-(phenylmethoxy)benzene, 129833-50-7; hydroxylamine sulfate, 10039-54-0; chloral hydrate, 302-17-0; 2-chloro-3-methyl-α-isonitrosoacetanilide, 129833-51-8; 3-fluoro-2-methyl-α-isonitrosoacetanilide, 114895-95-3; 3-bromo-2-methyl-α-isonitrosoacetanilide, 129833-52-9; 7-chloro-6-methylisatin, 129833-53-0; 6-chloro-7-methylisatin, 57817-03-5; 6-bromo-7-methylisatin, 129833-54-1; 4(α-isonitrosoacetamino)indane, 129833-55-2; 1,6,7,8-tetrahydrocyclopent[g]indole, 129848-59-5; methyl 6-(dimethylamino)-5-methyl-9-oxo-9H-xanthene-4-acetate, 129833-56-3.

Folate Analogues. 34. Synthesis and Antitumor Activity of Non-Polyglutamylatable Inhibitors of Dihydrofolate Reductase[1]

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Five analogues of methotrexate (MTX), 10-deazaaminopterin (10-DAM), and 10-ethyl-10-deazaaminopterin (10-EDAM) in which the glutamate moiety was replaced by either a γ-methyleneglutamate or β-hydroxyglutamate were synthesized and evaluated for their antifolate activity. These analogous are 4-amino-4-deoxy-N^{10}-methylpteroyl-β-hydroxyglutamic acid (1), 4-amino-4-deoxy-10-deazapteroyl-β-hydroxyglutamic acid (2), 4-amino-4-deoxy-N^{10}-methylpteroyl-γ-methyleneglutamic acid (3, MMTX), 4-amino-4-deoxy-10-deazapteroyl-γ-methyleneglutamic acid (4, MDAM), and 4-amino-4-deoxy-10-ethyl-10-deazapteroyl-γ-methyleneglutamic acid (5, MEDAM). None of these compounds were metabolized to the respective polyglutamate derivative as judged by their inability to serve as substrates for CCRF-CEM human leukemia cell folylpolyglutamate synthetase (FPGS) in vitro. All compounds inhibited recombinant human-dihydrofolate reductase (DHFR) at nearly equivalent magnitude as MTX. Growth-inhibition studies with H35 hepatoma, Manca human lymphoma, and CCRF-CEM human leukemia cells established greater cytotoxic effects with compounds 3–5 than with compounds 1 and 2. γ-Methyleneglutamate derivatives 3–5 were transported to H35 hepatoma cells better than MTX or β-hydroxyglutamate derivatives 1 and 2. Compound 3 was 2.5 times better than MTX in competing with folinic acid transport in H35 hepatoma cells. Compound 1 did not have a significant inhibitory effect on folinic acid transport even at 50 μM under identical conditions. The IC_{50} for compound 1 against H35-hepatoma cell growth was 8.5-fold higher than MTX. Compounds with the γ-methyleneglutamate moiety (3–5) exhibited almost equal or lower IC_{50} values than MTX against the growth of CCRF-CEM human leukemia cells. These studies show that on continuous exposure, the non-polyglutamylatable inhibitors DHFR (3–5) can exhibit superior antifolate activity compared to the polyglutamylatable methotrexate, presumably due to their enhanced transport to these cell lines. Compounds 3–5 appear to be excellent models to study the role of polyglutamylation of antifolates in antitumor activity and host toxicity.

The isolation and identification of the poly-γ-glutamyl metabolites of the well-known anticancer drug methotrexate (MTX) from human red blood cells were first reported in 1973.[2] With use of ^{14}C-labeled methotrexate, the formation of these metabolites in rodent tissues was subsequently confirmed.[3,4] Chemical synthesis of the poly-γ-glutamyl metabolites of methotrexate was accomplished by the solid-phase procedure, and these synthetic standards were utilized for the detailed study of their biochemical pharmacology[5,6] and role in methotrexate cytotoxicity.[5,6] Significant results of the investigations with synthetic MTX-polyglutamates that have therapeutic implications include (a) MTX polyglutamates inhibit dihydrofolate reductase (DHFR) and the IC_{50} values for DHFR inhibition decrease progressively as the number of glutamate residues is increased,[7] (b) the formation of MTX-polyglutamates in mammalian tissues is dose and time dependent,[8] (c) MTX-polyglutamates with longer chain lengths are retained longer within the cell,[8] (d) thymidylate synthase (TS) is inhibited more effectively by MTX-polyglutamates than the parent compound,[9] and (e) MTX-polyglutamates effectively inhibit aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFTase).[10] Like MTX, the antitumor agents 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin are also metabolized to their respective poly-γ-glutamyl derivatives in animal tissues.[11-13] Addition of γ-glutamate residues to 10-deazaaminopterin, 10-ethyl-10-deazaaminopterin,

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and methotrexate resulted in a dramatic increase in their TS inhibition.[12,13] Both normal tissues and tumors that can transport MTX at a faster rate will contain a larger fraction of the drug in the polyglutamate form and as free MTX will efflux from the cell, the polyglutamates replace the monoglutamate on DHFR and form a relatively stable pool of antifolate above the enzyme binding capacity.[14,15] These data taken together indicate that polyglutamylation of classical antifolates that are inhibitors of DHFR potentiates their cytotoxicity by inhibiting distal folate-based enzymes and permiting their longer retention in mammalian tissues.

The accumulation of methotrexate polyglutamates in normal proliferative tissues such as bone marrow, intestinal epithelial cells, liver, and kidney may contribute to the undesirable host toxicity of this drug and related antifolates. Therefore, it was of interest to synthesize a number of potent non-polyglutamylatable inhibitors of DHFR as potential anticancer drugs that may exhibit lower host toxicity without compromising their therapeutic effectiveness. Although a number of non-polyglutamylatable inhibitors of DHFR have been previously reported, many of these altered transport characteristics and inhibit folylpolyglutamate synthetase (FPGS) to varying degrees.[16] The most closely related analogue, fluoromethotrexate (FMTX),[17] has two asymmetric centers at the fluoroglutamate moiety, and consequently the separation of the four optical isomers is a tedious task. Indeed, the erythro diastereomer of fluoromethotrexate has been shown to be equally active as methotrexate in inhibiting CCRF-CEM and H35 hepatoma cells in culture.[17] The γ-methylene analogues of methotrexate and 10-deazaaminopterin (MMTX and MDAM) are racemic at the α-carbon and they exist as a mixture of two enantiomers. Compounds **1**, **2**, and **5** have two asymmetric centers and therefore are mixtures of diastereomers. As part of a continuing program aimed at the development of more specific and less toxic antifolates, in this paper we report the chemical synthesis and preliminary biological evaluation of certain non-polyglutamylatable analogues of methotrexate and 10-deazaaminopterins.

Chemistry

Polyglutamylation of folate or methotrexate is a process by which successive glutamate residues are added in a peptide linkage to the existing glutamate moiety through the γ-carboxyl group by the enzyme FPGS. The initial step in this metabolic transformation is thought to be the formation of a γ-acyl phosphate by reaction of the glutamate moiety of folate or methotrexate with ATP.[18] The acyl phosphate intermediate then reacts with the amino group of the incoming glutamate to form the γ-peptide bond with ejection of the phosphate. On the basis of this mechanism, we reasoned that introduction of the 3-hydroxy group at the glutamate moiety might prevent the formation of the acylphosphate by intramolecular hydrogen bonding of the β-hydroxyl with the γ-carboxyl group and the α-carboxyl group might remain free for enzyme binding and transport influx. Alternatively, introduction of the γ-methylene group at the glutamate moiety could interfere with polyglutamylation at the level of acyl phosphate formation by steric factors or by inhibiting FPGS via Michael addition of a nucleophile at the activity site of the enzyme with the initially formed acyl phosphate. Therefore compounds containing β-hydroxyglutamate and γ-methyleneglutamate were designed as potential non-polyglutamylatable antifolates in this study.

Table I. Inhibition of Recombinant Human Dihydrofolate Reductase by Compounds 1–5, MTX, 10-DAM, and 10-EDAM

compd	IC_{50},[a] nM	IC_{50} compound/ IC_{50} MTX
1	5.3	1.4
2	5.9	1.5
3	6.4	1.6
4	8.8	2.3
5	5.8	1.5
10-DAM	4.1	1.05
10-EDAM	4.4	1.13
MTX	3.9	1.0

[a] Assays performed as described in ref 24 with enzyme provided by Dr. J. H. Freisheim. The enzyme concentration in the assays was 8×10^{-9} M.

4-Amino-4-deoxy-N^{10}-methylpteroic acid (**6**), 4-amino-4-deoxy-10-deazapteroic acid (**7**) and 4-amino-4-deoxy-10-ethyl-10-deazapteroic acid (**8**) were synthesized as described.[3,19] D,L-3-Hydroxyglutamic acid was prepared according to the procedure of Vidal Cros et al.[20] 3-Hydroxyglutamic acid was converted to its dimethyl ester hydrochloride **13** by reaction with thionyl chloride in methanol. Diethyl D,L-4-methyleneglutamate hydrochloride (**19**) was prepared by reaction of commercially available methyl 2-(bromomethyl)acrylate with the anion derived from diethyl acetamidomalonate and hydrolysis of the resulting product (**16**) with 6 N HCl. D,L-4-Methyleneglutamic acid thus obtained was esterified with ethanol and thionyl chloride to **19**. Alternatively, sodium diethyl acetamidomalonate could be reacted with 2-(bromomethyl)acrylic acid in excess sodium ethoxide in ethanol to yield **17**, which could be subsequently hydrolyzed to D,L-γ-methyleneglutamic acid hydrochloride (**18**).

The pteroic acid analogues **6-8** were activated by converting them to their respective mixed anhydrides with isobutyl chloroformate[19] in the presence of triethylamine or *N*-methylmorpholine (Scheme I). The respective mixed anhydrides were then reacted with **13** or **19**, and the resulting products were hydrolyzed with 0.1 N NaOH to obtain compounds **1–5**. Compounds **1** and **2** were purified by DEAE-cellulose chromatography and **3-5** by reverse-phase chromatography on a C_{18} silica gel column.

Biological Evaluation and Discussion

Compounds **1–5** were tested for their ability to inhibit recombinant human dihydrofolate reductase. The results in Table I show that all the compounds were excellent inhibitors of human dihydrofolate reductase with similar degree of inhibitory potency. No significant differences were found among compounds with a 3-hydroxyglutamate or a 4-methyleneglutamate moiety in inhibiting this enzyme.

Substrate activities of both β-hydroxyglutamate-substituted (**1**, **2**) and γ-methyleneglutamate-substituted (**3–5**) compounds were compared to the activity of aminopterin with purified FPGS from CCRF-CEM human leukemia cells (Table II). Substrate activity as measured by [^{3}H]glutamate incorporation was <3% of that of aminopterin for all compounds. Thus, none of the compounds

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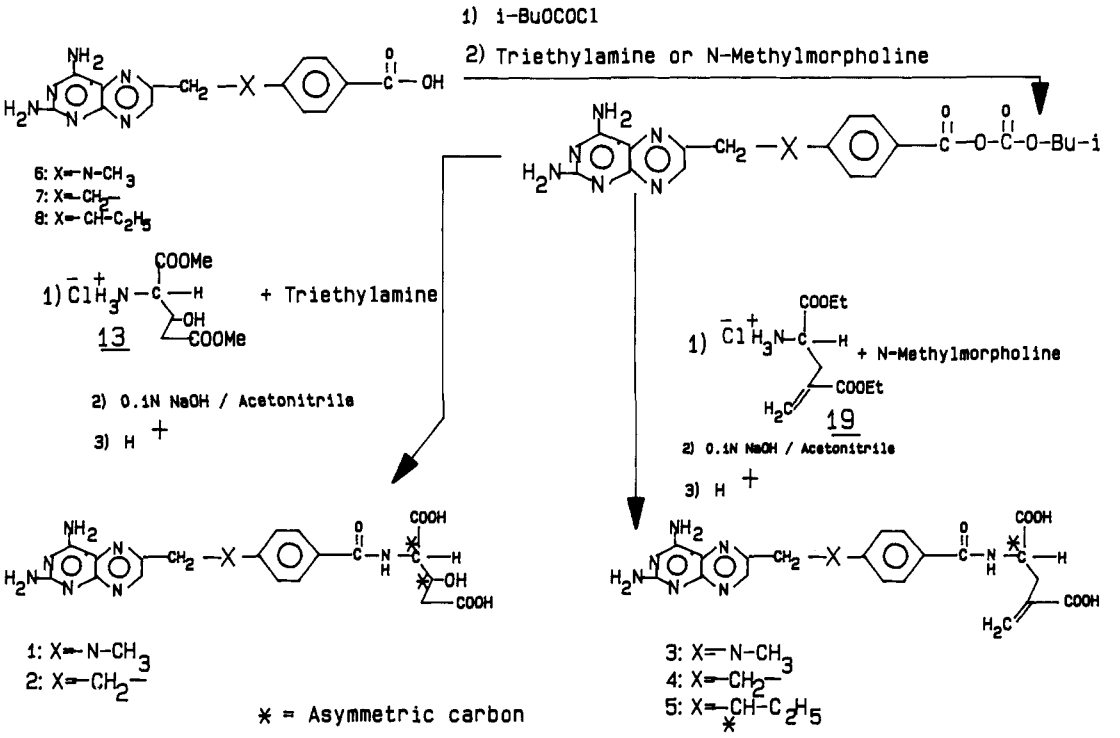
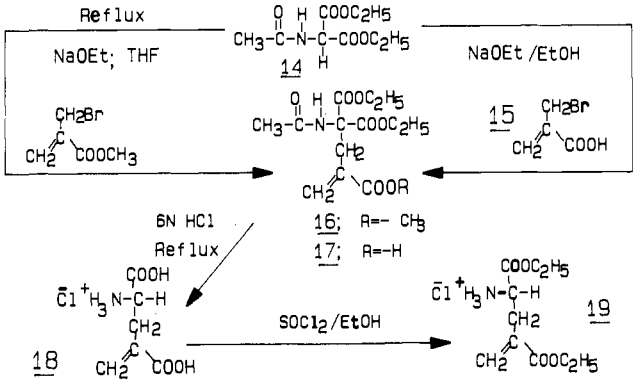
Scheme I



Scheme II



showed substrate activity, even at a concentration of 200 μM. These compounds were also tested as inhibitors of FPGS (Table II). Compound **2** was not inhibitory at 50 μM (the highest concentration tested) and compounds **1**, **3**, and **4** were extremely poor inhibitors (IC_{50} > 200 μM). Compound **5** was a weak inhibitor (IC_{50} = 118 μM). Thus β-hydroxy and γ-methylene substituents in the glutamate moiety of the antifolates led to complete loss of substrate activity and essentially complete loss of binding to FPGS.

The growth-inhibitory effect of compounds **1-5** was next evaluated with various tumor cell lines in culture. Results of continuous exposure of H35 hepatoma, Manca human lymphoma, and CCRF-CEM human leukemia cells to these compounds are shown in Table III. H35 cells that are resistant to MTX by virtue of a transport defect were cross-resistant to both hydroxyglutamate-substituted and 4-methyleneglutamate-substituted compounds. The γ-methyleneglutamate-substituted compounds **4** and **5** in the optically unresolved form were equally potent or more potent than MTX in inhibiting the growth of CCRF-CEM cells. Compound **5** exhibited exceptionally striking activity against all sensitive cell lines. If the D form is much less active[17,22] as is the case with MTX, then **5** will be 3–4 times more active (Table III) than MTX in inhibiting the growth

Table II. Substrate and Inhibitory Activity of Compounds 1–5 with CCRF-CEM Human Leukemia Cell FPGS[a]

	FPGS substrate activity		
compd	concn, μM	relative activity	FPGS inhibn: IC_{50}, μM
1	50	<3	≫200
2	50	<3	≫50
3	200	<1	≫200
4	200	<2	≫200
5	200	<1	118
MTX	50	38	
10-DAM	50	60	
aminopterin	50	100	

[a] Assays were performed as described in ref 25. Relative activity of each compound was compared to the activity of aminopterin at a concentration of 50 μM. Under these conditions aminopterin incorporated 800 pmol of [^{3}H]glutamate. Inhibition of FPGS was measured by adding increasing amounts of each compound to the assay mixture containing 50 μM aminopterin as the substrate.

Table III. Inhibition of Tumor Cell Growth by Compounds 1–5 and MTX[a]

	IC_{50}, nM			
compd	H35	H35R	Manca	CCRF-CEM
1	85 (10)	5600 (1800)	48 (6)	
2			29 (6)	
3	16 (10)	21000 (1800)	26 (6)	30 (10)
4	39 (10)	18400 (1800)	9.9 (5.2)	13 (14)
5	6.3 (10)	14000 (1800)	3.8 (5.2)	7.4 (14.5)

[a] Growth inhibition studies of H35 hepatoma cells were carried out as described in ref 26. H35R cells are ~100-fold resistant to MTX via a transport defect. Manca cell growth assay was performed according to ref 27. Detailed procedures of studies with CCRF-CEM human leukemia cells are described in ref 17. Values in parentheses are for methotrexate. Compounds **1**, **2**, and **5** were mixtures of diastereomers, whereas compounds **3** and **4** were racemic mixtures. No corrections were made for the presence of optical isomers of the D-methyleneglutamate or D-hydroxyglutamate moiety in any of the compounds (see refs 17 and 22).

Table IV. Growth Inhibition of H35 Hepatoma Cells after a 6-h Pulse[a]

compd	IC_{50}, μM
3	3.9
4	>20
5	14
MTX	0.085

[a] H35 cells were cultured for 24 h and the compounds added to the culture for 6 h (24–30). Cells were counted after 72-h exposure to the culture medium.

Table V. Inhibition of Folinic Acid Transport in H35 Hepatoma Cells by Compounds 1, 3, 4, 5, and MTX[a]

compd	IC_{50}[b] μM
1	no inhibn at 50 μM
3	7
4	5.5
5	1.5
MTX	18.0

[a] Transport experiments were done as described by Patil et al.[26] Folinic acid (2 μM, 15-min uptake). [b] The amount of compound required to effect 50% inhibition of the uptake of tritiated folinic acid during unidirectional influx and uncorrected for the presence of the D isomer of the methyleneglutamate moiety in any of the compounds.

of all MTX sensitive cell lines.

A 6-h-pulse growth-inhibition study was carried out with H35 hepatoma cells in culture (Table IV), and it was observed that MTX was 8-fold less effective than during continuous exposure. γ-Fluromethotrexate, a non-polyglutamylatable analogue of MTX, was 167-fold less potent than MTX on a 7-h pulse under identical conditions.[21] Analogues 3–5 were at least 2 orders of magnitude less toxic when a 6-exposure assay was done, and these results are consistent with the inability to form polyglutamates.[21]

γ-Methyleneglutamate-substituted compounds were found to be more potent than the 3-hydroxyglutamate-substituted compounds in inhibiting cell growth. Compound 1 was, however, more inhibitory to the MTX-resistant H35R hepatoma cells in culture. To determine whether differences in cell growth inhibition among the compounds were due to differences in their unidirectional transport influx, their transport characteristics were examined with the H35 hepatoma cells (Table V). On comparing the effects of 1 and 3 with that of MTX in competing for folinic acid transport, a significant difference was observed. MTX caused 50% inhibition of folinic acid transport at a concentration of 18 μM whereas a concentration of 50 μM of 1 did not have any significant inhibitory effect under identical conditions, indicating that 1 interacts only weakly with the reduced folate/MTX transport system. Compound 3 was a better inhibitor of folinic acid transport than MTX, exhibiting an IC_{50} value of 7 μM, compared to an IC_{50} of 19 μM for MTX. γ-Methylenemethotrexate (MMTX, 3) was considerably more cytotoxic to H35 cells than β-hydroxymethotrexate (1), indicating the importance of transport influx to cytotoxicity. Compounds 4 and 5 exhibited exceptional inhibition of folinic acid transport with IC_{50} values of 5.5 and 1.5 μM, respectively. Assuming that only those compounds with an L configuration at the glutamate moiety are activity,[22] the observed transport and cytotoxicity data with the γ-methylene antifolate 5 is quite remarkable. Compounds 1 and 5 have approximately the same polyglutamylation data and they are neither substrates nor inhibitors of CCRF-CEM human leukemia cell FPGS. However, compound 5 is more active than compound 1 in inhibiting cell growth (Table III), indicating that the enhanced transport of 5 may be responsible for its higher activity. Likewise, the diminished cytotoxicity of certain aspartate analogues of classical folate antagonists may not only be due to their poor substrate for FPGS but also their inefficient transport to mammalian cells.[23] This study has provided evidence that non-polyglutamylatable inhibitors of DHFR that are classical analogues of folic acid exhibit potent cytotoxicity if they are transported efficiently to tumor cells. Antifolate polyglutamates are retained in tumor and normal tissues for longer periods compared to their parent compounds. It is conceivable that accumulation of these metabolites in normal tissues may be partially responsible for the undesirable host toxicity exhibited by classical polyglutamylatable antifolates such as methotrexate. Since compounds 3–5 cannot be polyglutamylated, in vivo evaluation of one of these compounds might be useful in determining whether the lack of polyglutamylation of classical antifolates may provide any significant therapeutic advantage. Such studies are in progress.

Experimental Section

All organic solvents were dried over type 3A molecular sieves before use. Isobutyl chloroformate was freshly distilled and stored over $CaCO_3$. Triethylamine and *N*-methylmorpholine were redistilled prior to use. Ultraviolet spectra were obtained on a Bausch and Lomb Spectronic Model 2000 spectrophotometer interface with a Commodore Superpet computer. Melting points were determined on a Fisher Model 355 digital melting point analyzer. NMR spectra were run in $CDCl_3$ or CF_3COOH on a 90-MHz Perkin-Elmer Model R-32 spectrometer using Me_4Si as an internal standard unless otherwise specified. Field strength of the various proton resonances is expressed in parts per million and peak multiplicity is depicted as follows: s, singlet; br, broad singlet; d, doublet; t, triplet; q, quartet, c, unresolved multiplet, the center of which is given. HPLC analyses were carried out on a Waters 600A multisolvent delivery system equipped with a Model 481 UV detector and Waters 740 data module. Mass spectral analyses were determined by Dr. F. A. Bencsath, University of South Alabama, Mobile, AL.

Dimethyl D,L-Hydroxyglutamate Hydrochloride (13). 3-Hydroxyglutamate (12) was prepared according to the procedure of Vidal Cros et al.[20] To a stirring solution of 8 g (49 mmol) of 12 in 250 mL of dry methanol kept at 0 °C was added dropwise 25 mL of $SOCl_2$. The reaction mixture was stirred at 25 °C for 18 h and then refluxed for 4 h. Benzene (200 mL) was added to the reaction mixture and evaporated to dryness under reduced pressure to remove traces of H_2O and the product evacuated. The residue was dissolved in 100 mL of acetone, and upon slow addition of diethyl ether and trituration, crystals of 13 were formed. These crystals were separated by filtration, washed with 50 mL of ether followed by a small amount of acetone, and dried under vacuum over P_2O_5: yield 6.4 g (58%); mp 137-138 °C; NMR (TFA)

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δ 4.75 (br d, 1 H, α-H), 4.48 (c, 1 H, HCOH), 3.79, 3.65 (s, 3 H, carbomethoxy), 2.82 (d, 2 H, $-CH_2-$); MS m/z 192 (MH^+).

4-Amino-4-deoxy-N^{10}-methylpteroyl-β-hydroxyglutamic Acid (1). In a glass-stoppered measuring cylinder 325 mg (1 mmol) of 4-amino-4-deoxy-N^{10}-methylpteroic acid (**6**) prepared according to a literature procedure[4] was dissolved in 100 mL of dry DMF at 80 °C, and 0.28 mL (2 mmol) of triethylamine was added. After cooling of the solution to 0 °C in an ice bath, 0.262 mL of freshly distilled isobutyl chloroformate was added and the solution kept at this temperature for 90 min with occasional shaking. The reaction mixture was removed from the ice bath and set aside at 25 °C for 30 min. A solution of **13** (456 mg, 2 mmol) in 15 mL of DMF was neutralized with 0.28 mL of triethylamine, and it was added immediately to the above mixed anhydride solution and stirred for 18 h at 25 °C. The reaction mixture was evaporated to dryness at 55 °C under reduced pressure and stirred with a mixture of 10 mL of 5% $NaHCO_3$ and 10 mL of ether for 1 h. The suspension was filtered and the residue washed with water and hydrolyzed with a mixture of 80 mL of 0.1 N NaOH and 25 mL of acetonitrile for 18 h at 25 °C. After removal of the acetonitrile under reduced pressure, the pH of the solution was adjusted to 7.5 with 1 N HCl, diluted to 800 mL with distilled water and applied on a DEAE-cellulose column (chloride form). The product was eluted from the column using a linear NaCl gradient from 0.0 to 0.5 M in 0.005 M phosphate buffer at pH 7.0. Fractions corresponding to the desired product (**1**) were pooled, and the pH was adjusted to 3.5 with glacial HOAc. On refrigeration, a yellow precipitate of **1** was formed, which was filtered, washed, and dried in vacuum over P_2O_5: yield 180 mg (40%); mp 238–241 °C dec; MS (FAB) m/z 471 (MH^+) (calcd for $C_{20}H_{22}N_8O_6$ 470); UV λ_{max} (0.1 N NaOH) 256 nm (ε 22 870), 301 (ε 23 274), 370 (ε 7887); NMR (TFA) δ 2.75 (c, 2 H, γ-CH_2), 3.28 (s, 3 H, NCH_3), 4.51 (c, 1 H, CHOH), 4.8 (c, 1 H, α-CH), 5.0 (s, 2 H, CH_2NMe), 7.65 (q, 4 H, aromatic), 8.51 (s, 1 H, C_7H). Anal. C, H, N.

4-Amino-4-deoxy-10-deazapteroyl-β-hydroxyglutamic Acid (2). 4-Amino-4-deoxy-10-deazapteroic acid (**7**) was prepared by procedures previously developed in this laboratory.[19] Compound **7** (310 mg, 1 mmol) was dissolved in 100 mL of dry DMF by heating to 80 °C. The solution was cooled to 0 °C with an ice bath. To this stirring solution was added 0.14 mL (1.25 mmol) of *N*-methylmorpholine, followed by 0.13 mL (1 mmol) of isobutyl chloroformate. The reaction mixture was kept stirring at 0 °C for 25 min and then at 25 °C for 20 min. A solution of 456 mg (2 mmol) of compound **13** in 15 mL of DMF was neutralized with 0.225 mL (2 mmol) of *N*-methylmorpholine and immediately added to the above mixed anhydride (**10**) solution. The resultant reaction mixture was kept stirring for 18 h at 25 °C, heated to 55 °C, and evaporated to dryness. Crushed ice (50 g) was added to the residue, triturated, and filtered and the solid washed with water. This precipitate was stirred with 80 mL of 0.1 N NaOH at 25 °C for 18 h and the pH of the resultant solution was adjusted to 3.5 with 1 N HCl and chilled. The precipitate thus obtained was filtered and washed with water. HPLC analysis of the product indicated the presence of 54% of the product and 46% of unreacted pteroic acid. The crude product was dissolved in 0.1 N NaOH, and the pH of the solution was adjusted to 7.5–8 with 1 N HCl and purified by ion-exchange chromatography over DEAE-cellulose in the chloride form using a linear NaCl gradient from 0 to 0.5 M in 0.005 M phosphate buffer at pH 7.0 as described for **1**. The pure product was precipitated by acidifying the pooled fractions corresponding to the product to pH 3.5 with glacial HOAc. This mixture was chilled and the light yellow precipitate filtered, washed with water, and dried over P_2O_5 in vacuum: yield 177 mg (39%); mp 240–242 °C dec; UV λ_{max} (0.1 N NaOH) 254 nm, 369; MS (FAB) m/z 456 (MH^+) (calcd for $C_{20}H_{21}N_7O_6$ 455). Anal. C, H, N.

Diethyl D,L-4-Methyleneglutamate Hydrochloride (19). Diethyl acetamidomalonate (**14**) (6.1 g, 28 mmol) was added to a solution of 650 mg (28 mmol) of sodium metal in 50 mL of absolute ethanol and the reaction mixture was evaporated to dryness. Benzene (30 mL) was added to the residue and reevaporated twice to ensure complete removal of moisture. The above sodium diethyl acetamidomalonate was suspended in 65 mL of THF and 5 g (28 mmol) of methyl 2-(bromomethyl)acrylate (**15**) dissolved in 25 mL of THF was added. The reaction mixture was refluxed for 4 h and cooled to 0 °C with an ice bath which was followed by the addition of 14 mL of glacial HOAc. The mixture was evaporated to dryness under reduced pressure. After addition of 100 g of crushed ice to this residue, the mixture was extracted three times with EtOAc, and the combined EtOAc extracts were dried over Na_2SO_4. EtOAc was removed by evaporation, and the resultant residue (**16**) was refluxed for 18 h with a mixture of 80 mL of 6 N HCl and glacial HOAc (1:10) and evaporated to dryness. On evacuation, the oily residue (**18**) solidified. The solid was dissolved in 75 mL of dehydrated ethanol and cooled to 0 °C with an ice bath, and 14 mL of $SOCl_2$ was slowly added with stirring during a period of 30 min. The stirring was continued at 25 °C for 18 h, and the solution was refluxed for 3 h and evaporated after the addition of 200 mL of benzene to obtain a gummy compound. This gun was triturated with 15 mL of ether whereupon compound **19** was formed as white crystals, which were filtered, washed with 50 mL of ether, and dried under vacuum: yield 4.2 g (60%); mp 81–83 °C; MS m/z 215 (MH^+); NMR (TFA) δ 1.08 (c, 6 H, CH_2), 2.85 (c, 2 H, CH_2), 4.1 (c, 4 H, CH_2), 5.75, 6.26 (d, 2 H, olefinic).

5-Ethyl 4-Acetamido-4-(ethoxycarbonyl)-2-methyleneglutarate (17). To a stirring solution of 6.6 h (30 mmol) of diethyl acetamidomalonate (**14**) and 5 g (30 mmol) of 2-(bromomethyl)acrylic acid (**15**) in 50 mL of dry ethanol was added portionwise during a period of 1 h a solution of freshly prepared sodium ethoxide (90 mmol) under nitrogen. After overnight stirring (18 h), the reaction mixture was refluxed for 1.5 h and evaporated to dryness. The resultant residue was dissolved in 25 mL of ice-cold water and the pH of the solution adjusted to 4.0 with 6 N HCl. On refrigeration of the acidified solution, crystals of **17** were formed, which were filtered, washed with water, and dried in vacuum: yield 2.57 g (28%); mp 118–119 °C; NMR (TFA) δ 7.6 (s, 1 H, amide), 6.25, 5.52 (s, s, 2 H, methylene), 4.1 (c, 4 H, $-OCH_2-$), 3.17 (s, 2 H, CH_2), 1.9 (s, 3 H, acetamido), 0.98 (c, 6 H, CH_2CH_3); MS m/e 285. Anal. ($C_{13}H_{19}NO_6$) C, H, O. This compound was hydrolyzed with a mixture of HOAc and HCl to γ-methyleneglutamate hydrochloride (**18**) as described for the conversion of **16** to **18**.

4-Amino-4-deoxy-N^{10}-methylpteroyl-γ-methyleneglutamic Acid (3). To a solution of 326 mg (1 mmol) of 4-amino-4-deoxy-N^{10}-methylpteroic acid (**6**) in dry DMF was added 0.226 mL (2 mmol) of *N*-methylmorpholine and cooled to 0 °C. Isobutyl chloroformate (0.26 mL, 2 mmol) was then added and the solution stirred at this temperature for 30 min followed by another 30 min at 25 °C. A neutralized solution of **19** in 15 mL of DMF as described above was added to the mixed anhydride and the resultant reaction mixture was stirred for 18 h at 25 °C and evaporated to dryness at 55 °C under reduced pressure. The residue was triturated with 50 g of crushed ice, filtered, washed, and hydrolyzed with a mixture of 80 mL of 0.1 N NaOH and 25 mL of acetonitrile for 18 h at 25 °C. The pH of the solution was adjusted to 7.5 with 1 N HCl and concentrated to about 50 mL by rotary evaporation, and the crude product was precipitated by acidification of the concentrated solution to pH 3.5 with 1 N HCl. HPLC analysis revealed that the crude product was a mixture of 48% of **3** and 52% of unreacted pteroic acid (**6**). The crude product was purified on a column made of 15 g of C_{18} silica gel equilibrated with 10% acetonitrile in water. The product was eluted with 10% acetonitrile in water, the combined fractions containing the fast moving band were pooled, the pH was adjusted to 3.5 with glacial HOAc, and it was chilled. The precipitated product **3** was filtered, washed with water and dried over P_2O_5 under vacuum: yield 177 mg (38%); UV λ_{max} (0.1 N NaOH) 256 nm, 302, 371; MS (FAB) m/z 467 (MH^+) (calcd for $C_{21}H_{22}N_8O_5$ 466). Anal. C, H, N.

4-Amino-4-deoxy-10-deazapteroyl-γ-methyleneglutamic Acid (4). A solution of 310 mg (1 mmol) of 4-amino-4-deoxy-10-deazapteroic acid was cooled to 0 °C, followed by the addition of 0.226 mL (2 mmol) of *N*-methylmorpholine and 0.262 mL (2 mmol) of isobutyl chloroformate. After being stirred 30 min at 0 °C, the solution was stirred for 30 min at 25 °C and 503 mg (2 mmol) of neutralized **19** in DMF [neutralized with 0.226 mL (2 mmol) of *N*-methylmorpholine] was added. The resultant mixture was stirred for 18 h at 25 °C and evaporated to dryness under reduced pressure at 55 °C and the residue triturated with 50 g of crushed ice. The precipitated crude product was filtered and

stirred for 18 h with a mixture of 80 mL of 0.1 N NaOH and 25 mL of acetonitrile. The pH of the hydrolysate was adjusted to 7.5 with 1 N HCl, concentrated by rotary evaporation to about 25 mL, cooled to ~10 °C, and acidified with glacial HOAc to pH 3.5 and the resultant yellow precipitate was filtered, washed, and dried. HPLC analysis revealed that the precipitate was a mixture of unreacted pteroic acid (7) and the desired product 4. The crude product was dissolved in 15 mL of 5% NH_4OH and evaporated under reduced pressure to a residue which was dissolved in 10 mL of distilled water and applied on a column made of 15 g of C_{18} silica gel that was equilibrated with 10% acetonitrile in water. The product was eluted with 10% acetonitrile in water, the fractions containing the fast moving band on the column were pooled, and the pH was adjusted to 3.5 to precipitate product 4. The light yellow precipitate was separated by filtration, washed with distilled water, and dried: yield 180 mg (40%); mp 291-295 °C dec; UV (0.1 N NaOH) λ_{max} 254 nm (ϵ = 30704), 369 (ϵ = 6859); NMR (TFA) δ 2.95 (c, 6 H, CH_2), 4.82 (c, 1 H, CH), 5.74, 6.3 (d, 2 H, olefinic), 7.02, 7.39 (q, 4 H, aromatic), 8.37 (s, 1 H, C^7H); MS m/z 452 (MH^+) (calcd for $C_{21}H_{21}N_7O_5$ 451). Anal. C, H, N.

4-Amino-4-deoxy-10-ethyl-10-deazapteroyl-γ-methyleneglutamic Acid (5). The pteroic acid analogue 8 was prepared according to the procedure of Nair.[19] The mixed anhydride 11 was prepared exactly as described for 10 with 338 mg (1 mmol) of 8. After coupling of 11 with a neutralized solution of 19, the reaction product 5 was isolated and purified as described above for 4: yield 215 mg (45%); mp 253-257 °C dec; UV (0.1 N NaOH) λ_{max} 253 nm (ϵ = 31806), 370 (ϵ = 7491); MS (FAB) m/z 480 (MH^+) (calcd for $C_{23}H_{25}N_7O_5$ 479). Anal. C, H, N.

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Synthesis and Biological Activity of the 2-Desamino and 2-Desamino-2-methyl Analogues of Aminopterin and Methotrexate[1]

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The previously undescribed 2-desamino and 2-desamino-2-methyl analogues of aminopterin (AMT) and methotrexate (MTX) were synthesized from 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile. The AMT analogues were obtained via a three-step sequence consisting of condensation with di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate, heating with formamidine or acetamidine acetate, and mild acidolysis with trifluoroacetic acid. The MTX analogues were prepared similarly, except that 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile was condensed with 4-(*N*-methylamino)benzoic acid and the resulting product was annulated with formamidine or acetamidine acetate to obtain the 2-desamino and 2-desamino-2-methyl analogues, respectively, of 4-amino-4-deoxy-N^{10}-methylpteroic acid. Condenstion with di-*tert*-butyl L-glutamate in the presence of diethyl phosphorocyanidate followed by ester cleavage with trifluoroacetic acid was then carried out. Retention of the L configuration in the glutamate moiety during this synthesis was demonstrated by rapid and essentially complete hydrolysis with carboxypeptidase G_1 under conditions that likewise cleaved the L enantiomer of MTX but left the D enantiomer unaffected. The 2-desamino and 2-desamino-2-methyl analogues of AMT and MTX inhibited the growth of tumor cells, but were very poor inhibitors of dihydrofolate reductase (DHFR). These unexpected results suggested that activity in intact cells was due to metabolism of the 2-desamino compounds to polyglutamates.

The 2-amino group in the classical folic acid analogues methotrexate (MTX, 1) and aminopterin (AMT, 2) is generally assumed to be essential for biological activity; indeed, among the hundreds of AMT and MTX analogues synthesized and tested over the past 40 years, virtually all have retained the fused 2,4-diaminopyrimidine moiety, which is considered responsible for the tight binding of these molecules to the target enzyme dihydrofolate reductase (DHFR).[2] Analogues with the 2-amino group replaced by a 2-(dimethylamino)[3] or 2-methylthio[4] group were shown to have very little antifolate activity, supporting the concept that 2,4-diamino substitution is necessary for tight binding to DHFR. More direct evidence has been provided by X-ray crystallographic studies,[5-7] which reveal that the 2-amino group is hydrogen bonded to a highly conserved aspartic or glutamic acid residue in the active site of DHFR, as well as to a water molecule hydrogen bonded to a threonine residue. Replacement of the aspartic acid residue with serine by site-directed mutagenesis has been shown to produce a 3000-fold increase in the K_D for MTX binding as measured by equilibrium dialysis.[8,9] This substantial change corresponds to a decrease of 4.4 kcal mol^{-1} in the binding energy. In another study,[10] replacement of the threonine by valine, whose side

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